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14. ABSTRACT:

Our original hypothesis was "AKT regulates the RNA-induced silencing Complex (RISC) activity through the phosphorylation of AGO proteins in Prostate carcinoma cells". **Objectives:** To test the hypothesis following specific aims were proposed. **Aim 1:** To show that AKT regulates the miRNA expression profile in prostate carcinoma cells. **Aim 2:** To show that AKT mediated phosphorylation of AGO proteins regulates the activity of RISC complex. Aim 3: To show that abrogation of AKT mediated phosphorylation of AGO proteins sensitizes prostate carcinoma cells to chemotherapy. For Aim 1, we initially tried studying the interaction of Ago (pan) with 14-3-3. We could see interaction between Ago(pan) and τ (tau) isoform of 14-3-3. We treated prostate carcinoma cells (PC3) with 40 µM AKT inhibitor V, Triciribine.Total RNA was isolated and miRNA and mRNA expression analysis in prostate carcinoma cells (PC3) was carried out to identify the miRNA-mRNA correlation matrices using Pearson Correlation in response to cells treated with AKT inhibitor (Triciribine). We also identified the miRNA-mRNA correlation matrices using Pearson Correlation in response to cells treated with AKT inhibitor (Triciribine)These matrices were further classified into four groups viz., I. miRNA up mRNA down. II miRNA down mRNA up. III. Both miRNA and mRNA up. IV. Both miRNA and mRNA down..Using the GeneGo analysis we identified TGF-β dependent induction of Epithelial Mesenchymal Transition (EMT) via SMADs as its target. For aim 2, in order to identify the miRNA associated with specific AGO proteins, Immunoprecipitation was carried out.Since, the quality of miRNA isolated was poor, we could not proceed further on this aim. Once the miRNA-mRNA pair regulated by PI3K/AKT/14-3-3 is identified, we could then test the hypothesis that ***"AKT regulates the RNA-induced silencing Complex (RISC) activity through the phosphorylation of KSRP protein in Prostate carcinoma cells".***

15. SUBJECT TERMS
AGO, AKT, PI3K, miRNA, 14-3-3, KSRP

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Introduction

Prostate cancer (PCa) accounts for 29% of all diagnosed malignancies among men in the United States. An estimated 241,740 men will be diagnosed with PCA in 2013, and 28,170 men will die of it, making it the 2nd most common cause of cancer death{1} . The number of men ≥65 years is expected to increase 4-fold by 2050 {2}. These statistics portend a substantial increase in the number of men who will be diagnosed with PCa and who will require treatment for their malignancy. To control and target PCa, several signaling pathways need to be tested for therapeutic potential. One such pathway that has been implicated in prostate tumoriogenesis is PTEN/PI3K/AKT signaling pathway, Somatic *PTEN* alterations such as homozygous deletions,] Loss of Heterozygosity (LOH) and inactivating mutations, have been shown in both localized and metastatic PCa. Point mutations in primary tumors (1/40){3-4}, (5/37){5}; homozygous deletions (8/60){6}; LOH in 23/80 primary tumors with either deletion or mutation of the remaining allele in 10/23 LOH+ tumors was reported{7}. Thus, a substantial minority (~15%) of primary tumors harbors PTEN mutations. Formation of complex between AR, p85α and Src is required for androgen stimulated PI3K/AKT activation leading to cell survival{8}. Mutations of AR in PCa are rare in early stages, however, the frequency of mutations increase in the advanced, androgen independent tumors {9}. AR mutations lead to decreased specificity of ligand binding and inappropriate receptor activation by estrogens, progestins, adrenal androgens, glucocorticoids and/or AR antagonists. These mutations may still possess the ability to induce the PI3K/AKT activity. AR represses CaMKII which in turn repress the PI3K/AKT activity and loss of AR activity by AD results in induction of PI3K/AKT activity{10}. Thus, there are atleast two different mechanisms by which AR signaling regulates PI3K/AKT signaling pathway.

microRNAs (miRNAs), small non-coding RNAs, have emerged as key regulators of a large number of genes. The deregulation of the cellular "miRNome" in prostate cancer has been connected with multiple tumor-promoting activities such as aberrant activation of growth signals (such as PI3K/AKT signaling, activation of AR signaling), anti-apoptotic effects, prometastatic mechanisms, alteration of the androgen receptor pathway, and regulation of the cancer stem cell phenotype. With the elucidation of molecular mechanisms controlling and controlled by microRNAs, investigations are underway, in an attempt to exploit these molecules in the clinical setting. The knowledge gleaned from such studies could make the multifaceted biological activity of microRNAs an attractive candidate as anticancer agents{11}.

With advances in expression profiling microarray analysis on miRNAs has become fast and easy approach to detect distinctive signatures for specific tissues or disorders{12}. In Cancer, the association between miRNA and oncogene regulation has been reported and miRNA's involvement in cancers has been identified through microarray experiments{13}. Computational predictions estimate that miRNAs regulate about 60% of all human protein coding genes{14} affecting several cellular processes such as diffentiation, growth and apoptotis{15}. RNA silencing is a highly conserved gene regulatory mechanism present in almost all eukaryotic organisms. miRNA and SiRNA can inhibit gene expression in a sequence specific manner. Aberrant miRNA expression is a hallmark of several different types of cancer including Prostate cancer. miRNA inhibits the expression of many genes suggesting that comprehensive regulation can be achieved by antagonizing or overexpressing a single miRNA. We have uncovered a novel mechanism of miRNA processing by AGO family of silencing proteins through

PI3K/AKT signaling. Analysis of the several known components of miRNA biogenesis for consensus AKT phosphorylation and 14-3-3 binding sites (RSXpSXP and RXY/FXpSXP{16}) identified their presence in AGO1,3 and 4 ((in addition to KSRP and MOV10 protein); AGO1- rsffspp[183] and rsvsip[798]; AGO3- rsffsap[186] and rsvsip[801]; AGO4- rsffspp[175] and rsvsip[802]) but not Ago2 and Piwi family of proteins. AGO proteins are key players in RNA silencing pathways. Constitutive activation of PI3K/AKT signaling will therefore lead to phosphorylation of AGO proteins and loss, sequestration OR degradation of their activity. A dramatic increase in the levels of mature miRNA in response to increased expression of AGO proteins suggests that AGO proteins are limiting and serve to stabilize miRNA{17}. AGO2 is the only protein among the AGO family (which are components of RISC complex) of silencing proteins that has endonuclease (Slicer) activity, although all AGO proteins have a catalytic triad which is very close to the AKT phosphorylation site at the C-terminus. An intriguing question that emerges is that *"Does phosphoryation of AGO1, 3 and 4 by AKT regulate the function, localization, slicer activity and the stability of miRNA ?" Alternatively, does AKT activity cause a qualitative or a quantitative change in miRNA expression profiles in Prostate cancer cells?.* A schematic representation of this concept in shown in Figure 1.
It is hypothesized that ***"AKT regulates the RNA-induced silencing Complex (RISC) activity through the phosphorylation of AGO proteins in Prostate carcinoma cells".***

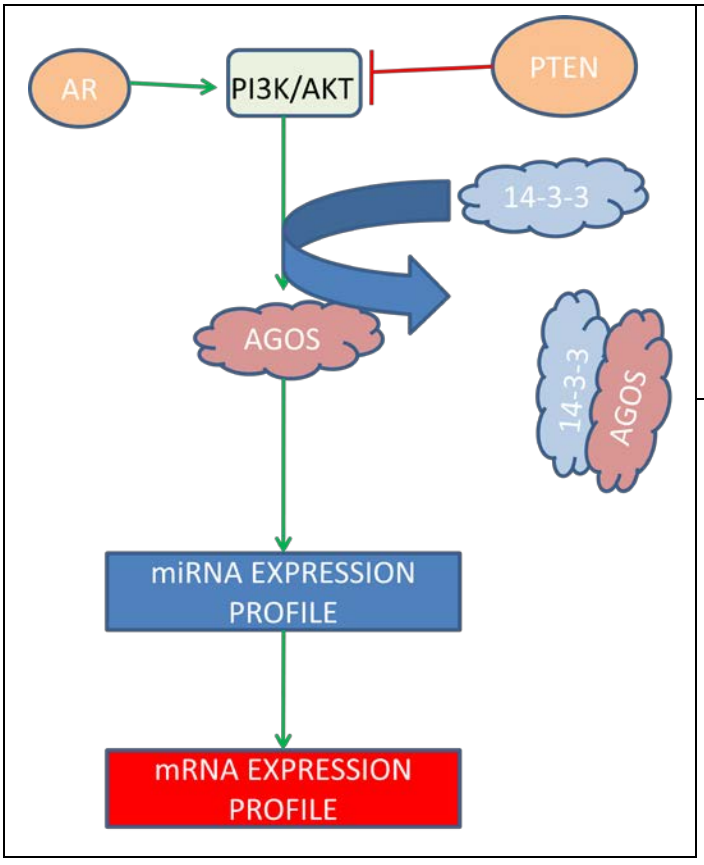

Figure 1. Schematic Representation of Role of PI3K/AKT signaling in miRNA/mRNA expression profiles. Loss of PTEN and/or Activation of AR leading to constitutive activation of PI3K/AKT will lead to phosphorylation of AGO proteins causing its interaction with 14-3-3 and possible 1.sequestration of RISC activity of AGO proteins. 2. Degradation of 14-3-3/AGO-protein/miRNA complex. Changes in miRNA expression profiles could effect the mRNA expression profiles.

Since we could not isolate miRNAs by immunoprecipitating the AGO protein complex we modified our hypothesis, KSRP is a single strand nucleic acid binding protein that controls gene expression at multiple levels. Binding of the human KSRP protein to let-7 miRNA precursors positively regulates their processing to mature let-7 (18). KSRP has an Akt phosphorylation site in its primary

structure which could be account for its effect on miRNA signaling. Thus now our hypothesis

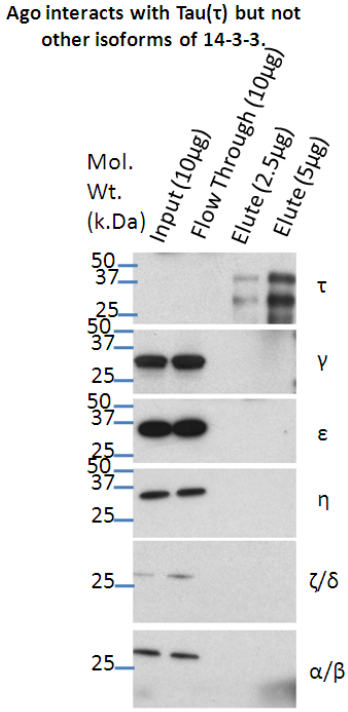

Figure 2. PC3 cell extracts were Immunoprecipitated with anti pan-AGO Antibody and the immunoprecipitates were Probed for the presence of different iso-Forms of 14-3-3. Out of all the isoforms of 14-3-3 only the Tau (τ) isoform of 14-3-3 Interacted with AGO proteins.

show if AGO proteins interact with 14-3-3 extracts of prostate carcinoma PC3 cells were immunoprecipitated with Pan-AGO antibody and the immunoprecipitate was probed with antibodies against different isoforms of 14-3-3 by Western immunoblot. Although we could not detect presence of Tau isoform of 14-3-3 in the input or flow through it was enriched in the elute (Figure 2). We still have to identify the isoform of AGO protein involved in this interaction.

AKT regulates expression of miRNA in Prostate carcinoma cells. In order to

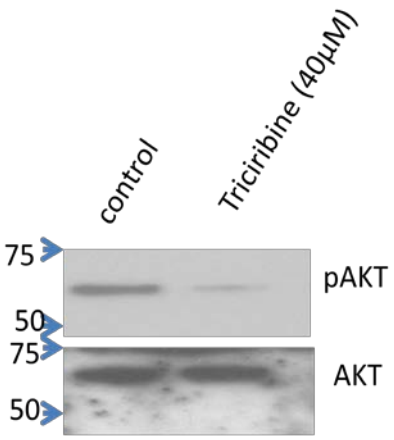

Figure 3.Triciribine inhibits AKT activity. Inhibition of AKT activity as refelected by loss of Serine474-AKT in cell treated with 40µM Triciribine. An antibody against phosphoserine 474 showed decrease in phosphorylation of phosphorylated and processing with AKt states that ***"AKT regulates the RNA-induced silencing Complex (RISC) activity through the phosphorylation of KSRP protein in Prostate carcinoma cells".***

Task 1: To show that AKT regulates the miRNA expression profile in prostate carcinoma cells **and Task2:** To show that AKT mediated phosphorylation of AGO proteins regulates the activity of RISC complex.

Interaction of AGO proteins with 14-3-3. In order

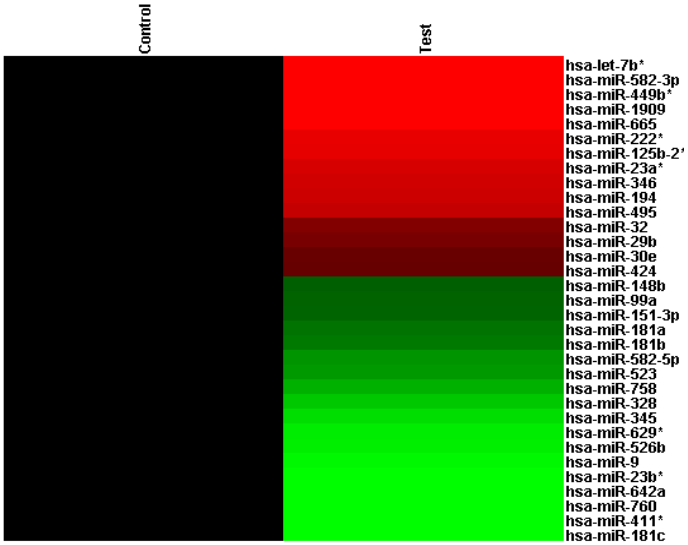

Figure 4. Heat map of MiRNA expression. Each miRNA listed was detected as significantly differentially expressed between control untreated PC3 cells and 40µM Triciribine treated PC3. As shown has-let7b* is the most up regulated miRNA and hsamiRNA 181c is the most down-regulated miRNA "Heatmap was generated using fold change differences between "Control" and "Test(TCN-trearted" groups"

to find out if AKT regulates the expression of miRNA,PC3 cells were treated with pan AKT inhibitor Triciribine. Triciribine is a potent, small-molecule inhibitor of activation of all three isoforms of AKT in vitro and in vivo. It was identified from a chemical library of 1,992 compounds from the National Cancer Institute Diversity Set for agents capable of inhibiting growth of AKT2-transformed but not parental NIH/3T3 cells{19} Of the 32 compounds that selectively inhibited growth of AKT2-transformed cells, the most potent was triciribine. Once inside cells triciribine(TCN) is converted to TCN-P by adenosine kinase{20}. Triciribine significantly inhibited AKT phosphorylation at both Thr309 and Ser474, which is required for full activation of AKT. Western immunoblot analysis of the extracts from PC3 cells either untreated (control) or treated with 40μM Triciribine showed loss of phosphorylation of AKT1 at Serine 474 (Figure 3.). Having confirmed the inhibition of AKT1 and possibly other isoforms (AKT2 and 3, as suggested in the literature, we carried out RNA extractions from the cells treated with 40μM Triciribine and untreated (control). Using the Agilent Bioanalyzer 2100 capillary electrophoresis system (Agilent, Santa Clara, CA, USA): A microfluidics-based platform for sizing, quantification and quality control of DNA and RNA, we analyzed the quality of RNA. The Bioanalyzer software produces a RIN (RNA Integrity Number) that allows a comparison of sample quality..

Microarray for expression of miRNA: Sixty nanograms of total RNA was reverse transcribed using the human Megaplex Primer Pools A and B and the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Each sample was pre-amplified for 12 cycles using human pool A and B Taqman® Megaplex™ PreAmp Primers and PreAmp Master Mix (Applied Biosystems) according to the manufacturer's instructions.

Table # 1 Control vs TN-trd	Fold change
hsa-miR-125b-2*	2.28231045434382
hsa-miR-222*	2.33655652450241
hsa-miR-629*	2.42455044682265
hsa-miR-526b	2.47549540559472
hsa-miR-9	2.51488956566013
hsa-miR-642a	2.53726257364516
hsa-miR-760	2.63585832208773
hsa-miR-665	2.73828541290375
hsa-miR-1909	2.74779205120459
hsa-miR-449b*	3.28030141020062
hsa-miR-411*	3.37122478824032
hsa-miR-181c	3.40645896370947
hsa-miR-582-3p	3.8192062363072
hsa-let-7b*	4.34173847372286

Table #2

Control vs TN-trd	Fold change
hsa-miR-328	-2.36481
hsa-miR-345	-2.61485
hsa-miR-629*	-2.7939
hsa-miR-526b	-2.82202
hsa-miR-9	-2.94186
hsa-miR-23b*	-3.00136
hsa-miR-642a	-3.14573
hsa-miR-760	-3.27553
hsa-miR-411*	-3.42516
hsa-miR-181c	-3.53913

Table#1 . List of miRNA which show up regulation with Akt inhibitor Triciribine.miRNA which show atleast 2-fold down regulation and a significance of atleast P≤ 0.05

Table# 2. List of miRNA which show down regulation with Akt inhibitor Triciribine.miRNA which show atleast 2-fold down regulation and a significance of atleast P≤ 0.05

For each sample, the preamplification reactions A and B were diluted and each reaction was combined with Taqman® Gene-Expression Master Mix (Applied Biosystems) divided into eight aliquots and each aliquot was added to one of the eight sample ports of the TaqMan® Array A or B (v2.0), respectively. The TaqMan® Array Human miRNA Card Set v2.0 enables detection of 667 human miRNAs, 3 miRNA endogenous reference controls and 1 miRNA assay not related to human as a negative control. The real-time polymerase chain reaction (PCR) reactions were run according to the manufacturer's instructions. RealTime Statminer Software (Integromics, Philadelphia, PA, USA) was used to analyze the data. The global geometric mean of all expressed miRNA assays was used to normalize the data.{21}. Of the ~300 miRNA showing changes when cells were treated with 40µM Triciribine and the miRNAs showing atleast two-fold change were selected with a significance of atleast $P \leq 0.05$ by Benjamini-Hochberg and Westfall & Young methods as shown in Figure 4 and Table 1 & 2. According to this analysis, let-7b* was the miRNA that was highly upregulated (4.34 folds in Triciribine treated PC3 cells compared to control at a significance of $p \leq 0.005$. Similarly hsa-miR-181c was the most down regulated miRNA in response to triciribine treatment in PC3 cells compared to control PC3 cells. (Table 1 & 2).

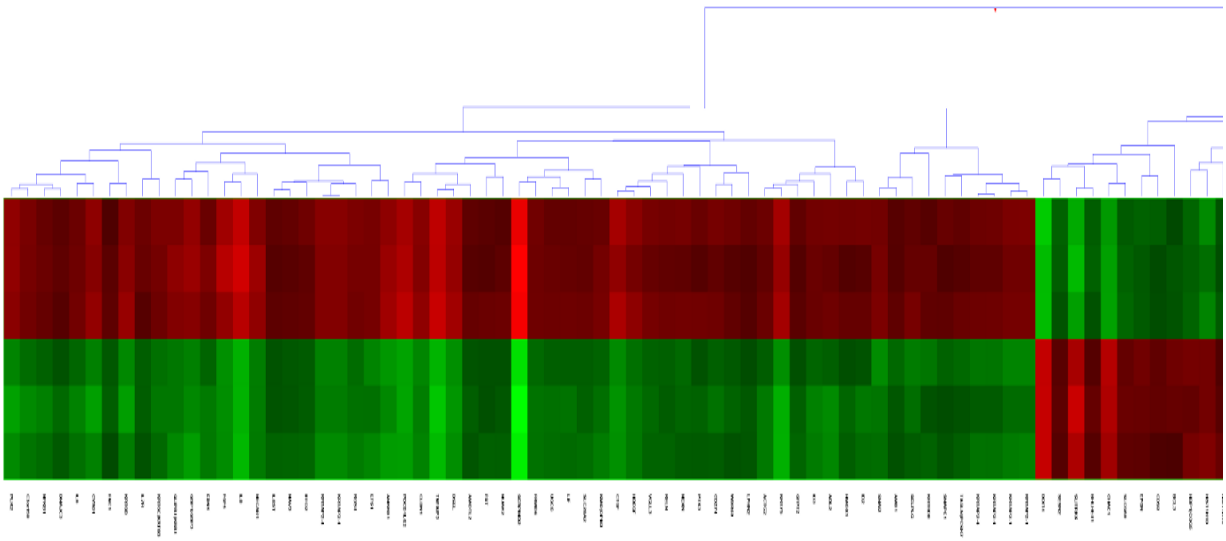

Figure 5. Heat map of mRNA expression. Each mRNA listed was detected as significantly differentially expressed between control untreated PC3 cells and 40µM Triciribine treated PC3 As shown DDIT4 is the most up regulated mRNA and SERPINB2 is the most down-regulated mRNA "Heatmap was generated using fold change differences between "Control" and "Test(TCN-trearted"

Microarray for expression of mRNA: Expression data obtained by hybridizing samples to HumanHT-12 V4_0_R1 array platform (Illumina) were loaded on GeneSpring® 12.5 GX (Agilent Technologies, Santa Clara, CA, USA).

The raw intensities from the array were normalized using '*Quantile normalization*' method and log (base 2) transformed. The normalized data was subjected to statistical analysis using paired Student's T-test (two-class comparisons). All significant and differentially expressed (DE) genes were subset using P-value ≤ 0.005 and fold change

≥ 2.0 thresholds. As shown in figure 5 and Table # 3 & 4, The, mRNA to show greatest up regulation was DDIT4(DNA damage-inducible transcript 4 protein) (12.173 folds) and the mRNA to show greatest down regulation was SERPINB2 (plasminogen activator inhibitor 2; 22.425 FOLDS). These lists of DE genes were analyzed for enriched pathways using the MetaCore™ software on GeneGo from Thomson Reuters.

Table # 3

genesymbol	Fold Change	Pvalue
DDIT4	12.173	0.001
SLITRK6	9.558	0.002
GDF15	8.679	0.002
CHAC1	8.266	0.001
LOC344887	5.867	0.005
GRB7	5.170	0.004
HIST1H1D	4.855	0.002
TCP11L2	4.811	0.004
CYP1A1	4.307	0.005
FLJ35776	4.288	0.002

Table # 4

genesymbol	Fold Change	Pvalue
SERPINB2	-22.425	0.002
TNFAIP3	-11.504	0.001
IL8	-11.104	0.001
PDCD1LG2	-8.690	0.001
KRT75	-8.010	0.002
FGF5	-7.070	0.002
CTGF	-6.981	0.002
ANKRD1	-6.841	0.002
DSEL	-6.804	0.001

Table# 3. List of mRNA which show up regulation with Akt inhibitor Triciribine.mRNA which show atleast 2-fold upregulation and a significance of atleast P≤ 0.005 are shown. **Table# 4. List of mRNA which show down regulation with Akt inhibitor Triciribine.** mRNA which show atleast 2-fold down regulation and a significance of atleast P≤ 0.005 are shown.

Correlation between miRNA and mRNA expression profiles to identify a potential model for regulation by PI3K/AKT/Ago/14-3-3 pathway. We carried out Chromatin IP assay for total AGO protein-associated miRNA.We found the quality of total RBA isolated to be very poor.Therefore this approach was abandoned. We took a different approach to identify miRNAassociated with PI3K/AKT signaling pathway. Since the results so far did not give the relationship network between miRNA and mRNA expression we sought to carry out a pearson correlation to identify a potential model to test the role of AKT-mediated phosphorylation of AGO proteins in differential miRNA processing in prostate carcinoma cells. All significant miRNAs (P-value (BenjaminiHochberg corrected) ≤ 0.05) were subset and searched against miRWalk database (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html) for predicted and validated microRNA targets {21}. Expression data for all the significant differentially expressed genes (P-value ≤ 0.05) among these gene targets for each miRNA were extracted from the Illumina gene expression microarray. The target genes were grouped into 4 categories (Up/down; Down/Up; Down/Down and Up/Up) depending on correlation to miRNA to mRNA expression patterns.{22}. Down/Down and Up/Up will probably be due to coregulation of miRNA and mRNA, either up or down regulation. Up/down and Down/Up will most likely be due to inverse correlation between miRNA and mRNA. We selected the most up regulated (HSA-miRNA-let7b 4.34) and down regulated miRNA (HAS-miRNA-181c;- 3.54) and identified the top 10

down regulated and up regulated mRNAs respectively by Pearsons correlation. (Table # 5 and 6). Using this strategy we could narrow down two networks viz.1. let7b-NFκB1 causing down regulation of NFkB1 with triciribine which could regulate NfκB signaling network. 2. let7b-cldn1 causing down regulation of claudin1 with triciribine which could regulate tight junctions in prostate carcinoma cells. These two pathways are being pursued. Thus, let-7b could regulate the survival(through NFκB) and metastasis (through claudin 1) of prostate carcinoma cells and could be a potential target for prostate cancer therapy. Using this strategy we eliminated miRNA and mRNA's that were coregulated either up or down.

Table # 5

miRNA	miRNA Fold Change	p-value	Target Gene Name	Evidence	mRNA Fold Change
let-7b*	4.34	0.00	CLDN1	Validated	-5.54
let-7b*	4.34	0.00	NFKB1	Validated	-3.96
let-7b*	4.34	0.00	HMOX1	Validated	-3.40
let-7b*	4.34	0.00	DDX6	Validated	-2.71
let-7b*	4.34	0.00	KITLG	Validated	-2.70
let-7b*	4.34	0.00	PDLIM5	Validated	-2.69
let-7b*	4.34	0.00	NP	Validated	-2.50
let-7b*	4.34	0.00	FERMT2	Validated	-2.50
let-7b*	4.34	0.00	CORO1C	Validated	-2.50
let-7b*	4.34	0.00	SRPRB	Validated	-2.49

TABLE #6 miRNA	miRNA Fold Change	Target Gene Name	Evidence	mRNA Fold Change
miR-181c	-3.54	ERBB2	Validated	1.74
miR-181c	-3.54	PML	Validated	1.59
miR-181c	-3.54	ALAS1	Validated	1.55
miR-181c	-3.54	EPOR	Validated	1.49
miR-181c	-3.54	HOXA11	Validated	1.43
miR-181c	-3.54	FASTK	Validated	1.36
miR-181c	-3.54	CDKN1B	Validated	1.23
miR-181c	-3.54	RASSF1	Validated	1.22
miR-181c	-3.54	TWIST1	Validated	1.22
miR-181c	-3.54	TNK2	Validated	1.21

Table #5: Top 10 mRNAs down regulated with increased expression of let7b in response to 40μM AKT inhibitor triciribine. Claudin1 (CLDN1) and NFκB (p50) mRNA were validated targets of let-7b down regulated by 5.54 and 3.96 folds.

Table #6: Top 10 mRNAs up regulated with decreased expression of miRNA-181c in response to 40μM AKT inhibitor triciribine. ERBB2(HER2/Neu) and PML(promyelocytic leukemia) mRNA were validated targets of miRNA-181c up regulated by 1.74 and 1.59 folds.

For the sake of time and expenditure we limited our studies to these two networks. We did not further explore miR-181c and its targets.

Experimental Validation of downregulation of CLDN1 and NFkBp50 by real time RT-PCR and Western blotting. We used quantitative Rt-PCR and western blot to validate changes seen in our microarray experiments. We used Pfaffl method {23} to quantify changes in the expression of Let7/b miRNA, CLDN1 and NFKB1(p50) mRNA. As seen with microarrays there was approximately 4.09 fold upregulation of let7b MiRNA and 4.25 and 3.466 fold down regulation of Claudin1 (CLDN1) and NFkB1 (p50) respectively(Figure 5).
In order to find out if the changes in expression seen with microarrays and quantitative RT-PCR translated into qualitative changes in protein expression of CLDN1 and NFKB1(p50) Western immunoblot was carried out. We found similar qualitative changes in the expression of CLDN1 and NFKB1(p50) both in the Du145 and PC3 cells suggesting a novel mechanism of regulation of these genes in prostate cancer.

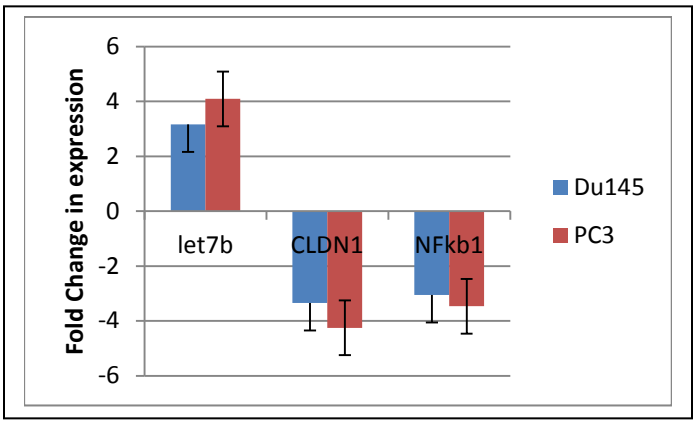

Figure 5. Fold Change in expression of Let7b miRNA, Cldn1 and NFkB1 (p50) mRNA. Control Du145 and PC3 cells and Du145 and pc3 cells treated with 40µM TCN were extracted for total RNA, reverse transcribed and specific miRNA and MRNA expression was detected by real time RT-PCR using the Pfaffl method. As seen with microarrays there was approximately 4.09 fold upregulation of let7b MiRNA and 4.25 and 3.466 fold down regulation of Claudin1 (CLDN1) and NFkB1 (p50) respectively.

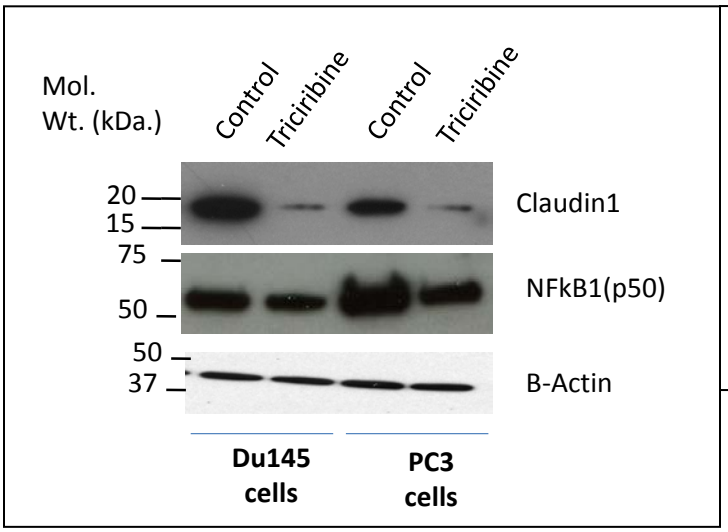

Figure 6. Fold Change in expression Cldn1 and NFkB1 (p50protein. Control Du145 and PC3 cells and Du145 and pc3 cells treated with 40µM TCN were extracted for protein and Cldn1 and NFkBp50 protein expression was assessed by Western immunoblotting. As seen with microarrays and real time RT-PCR there was down regulation of Claudin1 (CLDN1) and NFkB1 (p50) . β-actin used as an internal loading control did not show any change in expression.

Luciferase reporter to study the regulation of Let7b miRNA expression. In order to study the regulation of let7b expression we cloned the let 7b binding site into psiCheck 2.2. We can now study the regulation of let7b miRNA expression by different components of PI3K/AKT signaling in future.

Reportable Outcomes

This project started with the hypothesis ***"AKT regulates the RNA-induced silencing Complex (RISC) activity through the phosphorylation of AGO proteins in Prostate carcinoma cells".*** We could not get good quality miRNA using immunoprecipitation of total AGO proteins or by immunoprecipitation of biotynylated total RNA (including miRNA and mRNA). This led to a drastic change in our direction of research. Using the GeneGo analysis.we identified epithelial-mesenchymal transition as a mechanism that is affected with TCN-mediated inhibition of AKT signaling. . However, we could not confirm this mechanism in our studies. We hypothesized that altered miRNA will in turn effect the levels of mRNA targets. We treated prostate carcinoma cells (PC3) with 40 µM AKT inhibitor V, Triciribine.Total RNA was isolated and miRNA and mRNA expression analysis in prostate carcinoma cells (PC3) was carried out to identify the miRNA-mRNA correlation matrices using Pearson Correlation in response to cells treated with AKT inhibitor (Triciribine). We also identified the miRNA-mRNA correlation matrices using Pearson Correlation in response to cells treated with AKT inhibitor (Triciribine). These matrices were further classified into four groups viz., I. miRNA up mRNA down. II miRNA down mRNA up. III. Both miRNA and mRNA up. IV. Both miRNA and mRNA down.. Based on these analysis and latest findings in miRNA regulation (18)) and lack of obtaining results using AGO protein Immunoprecipitationswe came up with a model (Figure 7) wherein PI3K/AKT phosphorylates KSRP which can then bind to 14-3-3 zeta and sequesters it away from its function leading to repression of expression of Let7b miRNA which in turn downregulates the expression of CLDN1 and NFkBp50 mRNA(Figure 8). CLDN1 AND NFkb1(p50) mRNA are validated targets of Let7b (BLAT alignment against genome suggests intronic hits in both genes(Figure 7)

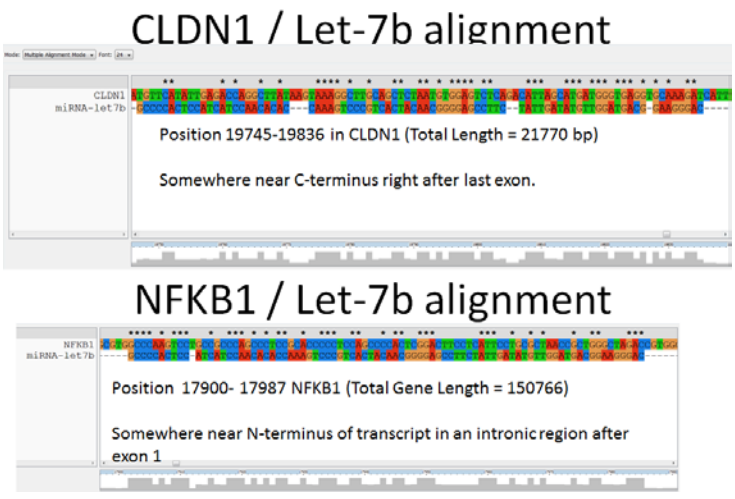

Figure 7. Using the BLAT sequence alignment we found let 7b targets in both CLDN1 and NFkB1(p50) mRNA.

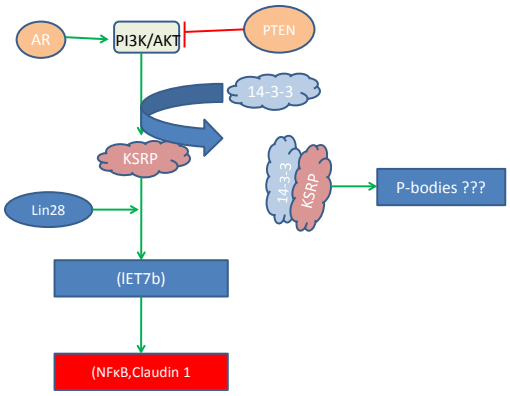

Figure 8. Schematic Representation of Role of PI3K/AKT signaling in miRNA/mRNA expression profiles. Loss of PTEN and/or Activation of AR leading to constitutive activation of PI3K/AKT will lead to phosphorylation of KSRP proteins causing its interaction with 14-3-3 and possible 1.sequestration of its activity of. 2. Degradation of 14-3-3/KSRP-protein/miRNA complex. Changes in miRNA expression profiles (Let7b) could affect the mRNA (CLDN1 and NFkB1(p50) mRNA expression profiles.

Key findings

1. Let7b miRNA was found to be upregulated with TCN treatment suggesting that PI3K/AKT signaling down regulates the expression of Let7b.
2. Using Pearson's correlation coefficient we found that Let 7b could target CLDN1 and NFkB1(P could target CLDN1 and NFkB1(p50). Indeed these are validated targets of let7b as also confirmed by BLAT analysis.
3. A model (Figure 8) is proposed for further studies.

We generated the following reagents during the course of this study

a. Psicheck-let7b reporter as described earlier.

Conclusions

In this study.we identified hitherto unknown mechanism of prosurvival strategies used by prostate cancer cells through the disruption of PI3K/AKT signaling mechanism. This involves miRNA-mRNA network., such as let7b-CLDN1 and let7b-NFkB1(p50) among a few pathways that are affected.

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